Expression and Function of *clpS* and *clpA* in *Xanthomonas Campestris* Pv. *Campestris*

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Abstract

ATP-dependent proteases (FtsH, Lon, and Clp family proteins) are ubiquitous in bacteria and play essential roles in many regulatory cell processes. *Xanthomonas campestris* pv. *campestris* is a Gram-negative pathogen and can cause black rot diseases in crucifers. The genome of *X. campestris* pv. *campestris* have several *clp* genes, namely *clpS*, *clpA*, *clpX*, *clpP*, *clpQ*, and *clpY*. Among them, only *clpX* and *clpP* were known to be required for pathogenicity. Here, we focused on two uncharacterized *clp* genes (*clpS* and *clpA*) that encode the adaptor (ClpS) and ATPase subunit (ClpA) of the ClpAP protease complex, respectively. Transcriptional analysis revealed that expression of both *clpS* and *clpA* is induced by heat shock. Inactivation of *clpA* but not *clpS* resulted in susceptibility to high temperature and revealed an attenuation of virulence on the host plant. The altered phenotypes of the *clpA* mutant could be complemented *in trans*. Site-directed mutagenesis revealed that K223 and K504 are critical amino acid residues for ClpA function in heat tolerance. The *clpA* mutant revealed different protein expression profiles as compared to the wild type in response to heat stress. Taken together, we characterized two *clp* genes (*clpS* and *clpA*) by examining their expression and function including stress tolerance and pathogenicity. We demonstrated that both *clpS* and *clpA* are expressed in a temperature dependent manner, and *clpA* is required for survival at high temperature and full virulence of *X. campestris* pv. *campestris*. This is the first time that *clpS* and *clpA* has been characterized in *Xanthomonas*.

Introduction

Regulated proteolysis is an essential process that affects several biological pathways (Mahmoud and Chien 2018). In bacteria, regulated proteolysis is carried out by energy-dependent AAA+ (ATPases associated with cellular activities) proteases that use the power of ATP hydrolysis to recognize, unfold, translocate, and degrade substrates (Mahmoud and Chien 2018). AAA+ proteases are not only required for regulated proteolysis but also essential for quality control of aberrant or aggregated proteins under certain conditions (Bittner et al. 2016).

Several AAA+ proteases exist in bacteria, such as FtsH, Lon, and Clp family proteases [ClpAP, ClpXP, and ClpYQ (HslUV)] (Bittner et al. 2016, Mahmoud and Chien 2018, Sauer and Baker 2011). All these AAA+ proteases are compartmentalized proteases that consist of two distinct functional units with separate activities: ATPase and protease (Baker and Sauer 2006, Bittner et al. 2016, Mahmoud and Chien 2018). In the case of FtsH and Lon, the ATPase domain and the protease domain are located on a single polypeptide (Bittner et al. 2016, Mahmoud and Chien 2018, Sauer and Baker 2011). In the Clp family, the AAA+ family ATPase subunit [ClpA, ClpX, and ClpY (HslU)] and the proteolytic subunit [ClpP and ClpQ (HslV)] are encoded in distinct polypeptide chains (Bittner et al. 2016, Mahmoud and Chien 2018, Sauer and Baker 2011). ClpA and ClpX function as chaperones and bind to ClpP (serine protease) to form the ATP-dependent proteases ClpAP or ClpXP, whereas ClpY (HslU) interacts with ClpQ (HslV) to form the ClpYQ (HslUV) protease (Chandu and Nandi 2004).

In the ClpAP protease, one or two AAA+ ClpA hexamers associated with the tetradodecameric ClpP to perform ATP-dependent protein degradation (Olivares et al. 2018). An adaptor protein may also be associated, such as ClpS, which interacts with the *N*-domain of ClpA and influences the ClpAP complex (Erbse et al. 2006). ClpAP exists in most Gram-negative proteobacteria and is characterized extensively in the model organism *Escherichia coli* (Kress et al. 2009a). ClpS is a highly conserved protein and ClpS homologs are found not only in bacterial but also in plant species (Dougan et al. 2002). As part of ClpAP complex, ClpA has several functions: ATP hydrolysis, substrate recognition, protein unfolding as well as translocation to its proteolytic partner ClpP (Xia et al. 2004). In the absence of the proteolytic component ClpP, ClpA functions as a molecular chaperone and catalyzes protein unfolding and limited protein remodeling (Wickner et al. 1994, Xia et al. 2004). ClpS is able to regulate ClpA substrate selection and is an essential component of the *N*-end rule pathway (Erbse et al. 2006).

Xanthomonas campestris pv. *campestris* is an important plant pathogen that causes a systemic vascular disease of crucifers known as black rot (An et al. 2020). Six predicted Clp family protein-encoding genes were annotated in the genome of *X. campestris* pv. *campestris* (da Silva et al. 2002, Liu et al. 2015, Qian et al. 2005, Vorholter et al. 2008). They are adaptor protein encoding gene (*clpS*), genes coding for AAA+ ATPase (*clpA*, *clpX*, and *clpY*), and genes coding for Clp protease (*clpP*, and *clpQ*). Among them, only two (*clpX* and *clpP*) have been studied. The *X. campestris* pv. *campestris* *clpX* was found to play an important role in bacterial attachment, stress tolerance, and virulence (Lo et al. 2020). The *X. campestris* pv. *campestris* *clpP* was showed to be required for growth under heat stress and in the presence of puromycin, essential for full virulence, and its expression was induced by heat shock (Li et al. 2020). In this study, we aimed to characterize *clpS* and *clpA* in *X. campestris* pv. *campestris*. We employed transcriptional fusion assay to evaluate the expression of *clpS* and *clpA*. In addition, we utilized deletion mutants of *clpS* and *clpA*, along with their genetic complements, to investigate the functions of the corresponding proteins in *X. campestris* pv. *campestris* stress tolerance and pathogenicity.

Materials And Methods

Strains, media, and culture conditions

The *E. coli* ECOSTM 101 Competent Cells [DH5a] used as the host for DNA cloning were purchased from Yeastern Biotech. The *X. campestris* pv. *campestris* wild-type strain Xcc17 was isolated in Taiwan (Yang and Tseng 1988). The Xcc17-derived mutant strains obtained by marker exchange included *DclpS* (*clpS* mutant) and *DclpA* (*clpA* mutant) were constructed in the present study. All bacteria cultures were routinely cultured in Luria–Bertani (LB) medium (Miller 1972), unless otherwise noted, and were grown at incubated 37 °C for *E. coli* and 28 °C for *X. campestris* pv. *campestris* strains, respectively. XOLN supplemented with glycerol (2%) was a basal salt medium (Fu and Tseng 1990). Ampicillin (50 µg/mL), kanamycin (50 µg/mL), gentamycin (15 µg/mL), and tetracycline (15 µg/mL) were added to the medium as required. For liquid cultures, bacteria cells were grown with shaking at 180 revolutions per minute (rpm). The agar concentration used for solid media was 1.5%.

Molecular techniques

The restriction enzymes, T4 DNA ligase, and Taq DNA polymerase used in this study were purchased from Promega, Roche, and Yeastern. DNA manipulation techniques and DNA transformation of *E. coli* were performed using standard methods as described (Sambrook et al. 1989). Genomic DNA and plasmid DNA were prepared using the Wizard[®] Genomic DNA Purification Kit (Promega) and the Gene-Spin[™] Miniprep Purification Kit (Protech), respectively. The polymerase chain reaction (PCR) was performed as previously described (Hsiao et al. 2005) using the primers listed in **Table 1**. DNA sequences were determined by Mission Biotech Co., Ltd. (Taipei, Taiwan). Transformation of *X. campestris* pv. *campestris* was carried out by electroporation (Wang and Tseng 1992).

Upstream region construction and promoter activity assay

DNA fragments containing the upstream regions of *clpS* and *clpA* were PCR-amplified using primer pair 35PstI/476XbaI and 621PstI/946XbaI, ligated into cloning vector yT&A (Yeastern), generating pTclpS and pTclpA. After sequence confirmation, the insert in pTclpS and pTclpA was excised and cloned ahead of a promoterless *lacZ* gene (reporter) in pFY13-9 (Lee et al. 2001), giving pFYclpS and pFYclpA. The resulting plasmids carried nt -448/-7 (442 bp) and -326/-1 (326 bp) region relative to the *clpS* and *clpA* putative translational start point, respectively (**Fig. 1**).

For promoter activity assay, the *X. campestris* pv. *campestris* wild-type Xcc17 carrying these constructs were cultured overnight and inoculated into fresh media to obtain an initial optical density at 550 nm (OD₅₅₀) of 0.35. Then, the cultures were incubated either at 28 °C or at 37 °C. Aliquots were withdrawn at 6, 24, 30 and 48 h post-incubation and the b-galactosidase activity was determined as described previously (Miller 1972). The experiment was performed in duplicate and repeated at least three times. Data represent the average of three replicates.

Sequence analysis and homology modeling

The nucleotide and the predicted amino acid sequence as well as the sequence comparison were analyzed online (<http://www.ncbi.nlm.nih.gov>). The three-dimensional structural model of Xcc17 ClpS and ClpA was based on the *E. coli* strain K-12 ClpS (PDB ID 2W9R) and ClpA (PDB ID 1KSF) from RCSB PDB database. They shared 56.4% and 63.0% sequence identity with Xcc17 ClpS and ClpA, respectively. The homology modeling structure of Xcc17 ClpS and ClpA used the project mode with the SWISS-MODEL workspace (<https://swissmodel.expasy.org>) (Arnold et al. 2006). The web-based application of NGL viewer (<http://proteininformatics.charite.de/ngl>) was used for the molecular visualization of protein structure.

Mutant construction and complementation

To construct the *clpS* mutant, the 135 bp *PstI-EcoRI* fragment in pTclpA was excised and cloned into pOK12 (Vieira and Messing 1991). The obtained plasmid pOKclpS, containing the internal region of the

Xcc17 *clpS* gene, was then transferred into Xcc17 by electroporation following a single crossover. For the *clpA* mutant construction, the 2334-bp *HindIII-XbaI* fragment encompassing the upstream 48 bp fragment plus the entire coding region of the Xcc17 *clpA* was synthesized and cloned in pUC57-Mini (Protech), giving pUCclpA. Then, the 693-bp *EcoRV-PstI* fragment of pUCclpA was excised and cloned into pOK12, giving pOKclpA. A Gm^R cartridge from pUCGM (Schweizer 1993) was inserted into the *HindII* site within the pOKclpA insert. The resultant plasmid, pOKclpAG, was further transferred into Xcc17 by electroporation allowing for double crossover. Insertion of pOKclpS or Gm^R cartridge into the target gene (*clpS* or *clpA*) was verified by PCR. The confirmed *clpS* and *clpA* mutant strains were designated as *DclpS* and *DclpA*.

For construction of the *DclpS* complementation plasmid, the 546 bp DNA fragment encompassing the upstream 82 bp fragment plus the entire coding region of the Xcc17 *clpS* was PCR amplified using primer pair 402HindIII/946XbaI and cloned into the broad-host-range vector pRK415 (Keen et al. 1988), giving pRKclpS (**Fig. 1**). For construction of the *DclpA* complementation plasmid, the 2334-bp *HindIII-XbaI* fragment of pUCclpA was excised and cloned in pRK415, yielding pRKclpA (**Fig. 1**). For complementation of the *clpS* and *clpA* mutants, plasmid pRKclpS and pRKclpA were electroporated into *DclpS* and *DclpA*, respectively. The complemented strains were designated as *DclpS*(pRKclpS) and *DclpA*(pRKclpA). In parallel, empty vector pRK415 was introduced into Xcc17, *DclpS* and *DclpA*, generating Xcc17(pRK415), *DclpS*(pRK415) and *DclpA*(pRK415) for comparison.

Site-directed mutagenesis

The site-directed mutagenesis of ClpA was carried out by the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies), according to the manufacturer's instructions. The mutation was constructed in the most commonly mutated residue in the Walker A motif of AAA⁺ protein (Wendler et al. 2012). The invariant lysine at position 223 and 504 was substituted to glutamine, using pUCclpA as a template for PCR and the primers listed in **Table 1**. After DNA sequence verification, the mutated *clpA* was cloned into pRK415 to give pRKclpAK223Q and pRKclpAK504Q (**Fig. 1**). These resulting constructs were separately introduced into *DclpA*, yielding *DclpA*(pRKclpAK223Q) and *DclpA*(pRKclpAK504Q).

Temperature tolerance assay

The method of temperature tolerance assay was as described earlier (Lo et al. 2020). Briefly, overnight cultures of tested strains were diluted with fresh LB broth to an initial density of OD₅₅₀ = 0.5 (5×10^8 cells/mL) and followed tenfold serial dilutions (5×10^7 to 5×10^5 cells/mL). Then, cell suspensions (5 mL) from each dilution were spotted on LB plates and incubated either at 28 °C or at 37 °C for 4 days. The assay was repeated at least three times, independently, in two replicates for each time.

Cell extracts preparation and analysis

Overnight grown cultures of tested strains were inoculated into fresh XOLN-glycerol medium to obtain an initial OD₅₅₀ = 0.35 and incubated at either at 28 °C or at 37 °C for 24 h. Bacteria cells were collected

through centrifugation at 12,000 × *g* for 2 min at 4 °C. Each preparation was obtained from the same number of cells. The cell pellets were rinsed and suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Cell extracts were obtained through sonication (cycles of 4 sec pulse and 5 sec rest on ice for 2 min). Following sonication, the cell debris and unbroken cells were removed by centrifugation at 2,000 × *g* for 2 min at 4 °C, and the supernatant fraction was referred to cell extracts.

Protein aggregates were isolated according to a procedure described previously (Kthiri et al. 2010) with some modifications. In brief, cell extracts were incubated with or without 0.5% Triton X-100 for 15 min at 25 °C. The insoluble cell fractions were recovered by subsequent centrifugation at 8,000 × *g* for 15 min at 25 °C. The insoluble cell fractions treated with Triton X-100 were referred to protein aggregates, and which omitting Triton X-100 treatment was obtained for comparison. The insoluble cell fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and protein bands were stained with Coomassie brilliant blue. The amount of loading was normalized in terms of colony forming units (CFUs) and was equivalent to 4.2×10^8 CFUs/lane.

Virulence assay

The virulence of *X. campestris* pv. *campestris* strains on cabbage was determined using the leaf clip inoculation method (Hsiao et al. 2011). Leaves were cut with scissors dipped in the bacterial suspension with cell concentration adjusted to OD₅₅₀ = 1. Lesion lengths were measured and images were taken 14 days post-inoculation. Three independent experiments with six replicates were performed. The values are presented as mean lesion length ± standard deviations.

Results And Discussion

Expression of *clpS* and *clpA* is induced by heat treatment

The genome of the sequenced *X. campestris* pv. *campestris* strain 17 (Liu et al. 2015) possesses *clpS* (locus_tag AAW18_RS09760) and *clpA* (locus_tag AAW18_RS09765), which adjacent to each other on the chromosome (**Fig. 1**). Genes *infA* and *aat*, coding for translation initiation factor IF-1 and leucyl/phenylalanyl-tRNA-protein transferase, are located downstream of *clpA*. As shown in **Fig. 1**, there are such compatible homologs with 49-69% shared amino acid sequence identities in *X. campestris* pv. *campestris* strain 17 and *E. coli* strain K-12 (Blattner et al. 1997). The *mutT* gene (locus_tag AAW18_RS0975 in *X. campestris* pv. *campestris* strain 17), coding for NUDIX hydrolase, is located upstream of *clpS*, and its homologue appears elsewhere in the chromosome in *E. coli* K-12 (Blattner et al. 1997). In addition to *X. campestris* pv. *campestris* strain 17, the organization of *clpS* and *clpA* as well as their flanking genes is similar in several *X. campestris* pv. *campestris* strains, such as ATCC33913, 8004, and B100 (da Silva et al. 2002, Qian et al. 2005, Vorholter et al. 2008).

Gene organization analysis showed that *clpS* and its upstream gene *mutT* as well as *clpA* and its downstream gene *infA* are in the opposite direction, and the *clpS* and *clpA* genes are linked together in the same orientation with 143 bp intergenic space (**Fig. 1**). Owing to its orientation and intergenic regions

with flanking genes, *clpS* and *clpA* are likely to possessing their own promoter. Sequence analysis revealed that the upstream regions of *clpS* and *clpA* shown to resemble the *E. coli* s^{32} -dependent heat shock promoter (TTGAAA-N₁₃₋₁₄-CCCCATNT) (Koo et al. 2009, Nonaka et al. 2006). A possible s^{32} promoter with a –35 box (CGTAAA) and a –10 box (GGCCAAAT) were located at –61 and –39 (with a spacer of 14 nucleotides) relative to the *clpS* translation start site. A putative s^{32} -type promoter with –35 box (TGGAAA) and a –10 box (CCGCATAT) separated by 13 nucleotides was found 29 nucleotides upstream of the *clpA* initiation codon. The presence of a s^{32} -type promoter led us to predict that the expression of *clpS* and *clpA* may be influenced under heat shock condition.

To evaluate the expression of *clpS* and *clpA*, the upstream regions of *clpS* and *clpA* were cloned ahead of a promoterless *lacZ* gene in pFY13–9 (Lee et al. 2001) as described in the **Materials and methods**, yielding pFYclpS and pFYclpA. Then, the generated reporter constructs were introduced into Xcc17 and the resulting strains, Xcc17(pFYclpS) and Xcc17(pFYclpA), were used to evaluate the expression of *clpS* and *clpA*. The promoter activities of *clpS* and *clpA* were assayed by measuring the b-galactosidase activities in Xcc17(pFYclpS) (**Fig. 2a**) and Xcc17(pFYclpA) (**Fig. 2b**) grown at 28 °C or 37 °C. When strains grown at normal physiological temperature (28 °C), the activities measured in Xcc17(pFYclpS) and Xcc17(pFYclpA) were 69 U and 121 U at 6 h, increased following cell growth, and gave highest level at 48 h (343 U and 769 U). When strains grown at 37 °C (heat stress condition), the b-galactosidase activities levels increased gradually and reached their maxima at 48 h, which were 413 U in Xcc17(pFYclpS) and 1220 U in Xcc17(pFYclpA), respectively, exhibiting approximately 1.6-fold increment in both cases compared with normal growing temperature. Results from reporter assays indicated that the expression of *clpS* and *clpA* is growth phase dependent and subjected to heat shock induction. Limited information was available regarding the expression of *clpS* and *clpA*, and the effects of heat stress on their expression is not similar among bacteria. The cellular level of ClpA in *Vibrio vulnificus* was induced under heat shock condition (Lee et al. 2018). In *E. coli*, the *clpA* gene has been reported as a monocistronic message whereas the synthesis of ClpA does not increase on heat shock (Gottesman et al. 1990, Katayama et al. 1988). In the case of *Brucella suis*, the expression of *clpA* is not increased at high temperature (Ekaza et al. 2000). It is indicated that PhoP represses the transcription of *clpS* but not *clpA* in *E. coli* and *Salmonella enterica* when bacteria experience a low cytoplasmic Mg²⁺ concentration (Yeom et al. 2018). Recently, it is reported that *clpS* and *clpA* is induced during H₂O₂ stress and OxyR is required for H₂O₂-driven induction of *clpSA* in *E. coli* (Sen et al. 2020). The effects of OxyR and PhoP on the expression of *clpS* and *clpA* in *X. campestris* pv. *campestris* remains to be elucidated.

Bioinformatics analyses indicate that ClpS and ClpA retain key residues required for their functionality

The *clpS* open reading frame is 321 bp in length and located in the genome sequence at position 2227483–2227803 in the Xcc17 chromosome (GenBank accession number NZ_CP011946). The predicted protein encoded by *clpS*, which consists of 106 amino acids and is annotated as an ATP-dependent Clp protease adaptor ClpS, has a calculated molecular mass of 12601 Da and a pI of 6.03. Domain organization analysis indicated that it has a ClpS domain situated at residues 23–102 (bit score:

113.20, E-value: 1.1e-29) (**Fig. 3a**). A BLAST search against proteins deposited in the Protein Data Bank (PDB) with the amino acid sequence of Xcc17 ClpS revealed that *E. coli* ClpS (PDB ID 2W9R) has highest homology with the Xcc17 ClpS (56% Identities, 81% Positives). The predicted three-dimensional structure of Xcc17 ClpS using *E. coli* ClpS (PDB ID 2W9R) as template was shown in **Fig. 3b**. The amino acid residues contributed to the interaction of ClpS and the *N*-domain of ClpA in *E. coli* (Guo et al. 2002, Xia et al. 2004) are conserved in Xcc17 ClpS and are situated at P24, P25, Y28, E79, E82, and K84 (associated with contact A of ClpA), and D36 and Y37 (associated with contact C of ClpA) (**Fig. 3b**). These observations suggested that, similar to situation found in *E. coli*, the Xcc17 ClpS may complex with *N*-domain of ClpA and function as a modulator of ClpA.

The *clpA* gene is 2283 bp in length and is located at position 2227947–2230229 in the Xcc17 chromosome (GenBank accession number NZ_CP011946). It is predicted to encode an ATP-dependent Clp protease ATP-binding subunit ClpA, which consists of 760 amino acids with a calculated molecular mass of 83346 Da and a pI of 5.66. Domain analysis revealed that Xcc17 ClpA consists of three functional domains: one *N*-domain and two AAA+ ATPase domains (designated as AAA+ modules D1 and D2) which are each divided into a large sub-domain and a small sub-domain (**Fig. 4a**). A BLAST search against the PDB with the amino acid sequence of Xcc17 ClpA revealed that ClpA from *E. coli* (PDB ID 1KSF) has the highest identity with the Xcc17 ClpA (64% Identities, 79% Positives). The predicted Xcc17 ClpA three-dimension structure built by homology modeling using *E. coli* ClpA as template structure is shown in **Fig. 4b**. In *E. coli*, the *N*-domain of ClpA has multiple ClpS binding sites (Xia et al. 2004) and is essential for docking of ClpS and required for the recognition, and degradation of some substrates (Dougan et al. 2002, Erbse et al. 2008, Guo et al. 2002, Zeth et al. 2002). Several amino acid residues contribute to the interactions of the *N*-domain of ClpA and the ClpS in *E. coli* (Xia et al. 2004) are highly conserved in Xcc17 ClpA. They include residues participating in ClpS binding via contact A (E23, F24, V27, E28, T81, R86, D116, and Y121) and residues involved in ClpS binding via contact C (S97, K99, N106, R130, and H139), respectively. In addition, three residues (H22, H29, and E63) in Zn²⁺ binding motif and two residues (Y14 and F84) in the *N*-domain hydrophobic path for peptide binding are also conserved in Xcc17 ClpA. These amino acid residues in the built three-dimension structure of ClpA are shown in **Fig. 4c** (left part). The ATPase domain of AAA+ protease consists of several classical key elements of ATPase function forming the nucleotide binding pocket, including Walker A, Walker B, sensor 1, arginine finger, and sensor 2 (Bittner et al. 2016, Miller and Enemark 2016, Wendler et al. 2012). The Walker A motif (GxxxxGKT/S, where x is any amino acid) is required for ATP binding and oligomerization whereas Walker B motif (hhhhDE, where h is any hydrophobic amino acid) is essential for ATP hydrolysis (Bittner et al. 2016, Miller and Enemark 2016). Both sensor 1 and arginine finger motifs are required for ATP hydrolysis whereas sensor 2 mediates conformational changes associated with a cycle of ATP binding and hydrolysis (Miller and Enemark 2016). These sequence motifs are highly conserved in both AAA+ modules D1 and D2 in Xcc17 ClpA. The Walker A motif in D1 and D2 is situated in 217–224 (GEAGVGKT) and 498–505 (GPTGVGKT), respectively. The Walker B motif in D1 and D2 is situated in 284–289 (VLFIDE) and 562–567 (VLLLDE). The sequences and positions of sensor 1, arginine finger, and sensor 2 motifs in D1 and D2 are as follows: (i) C³²¹IGSTT³²⁶ and L⁶⁰⁴VMTTN⁶⁰⁹ (sensor 1), (ii) R³⁴²R³⁴³ and

R⁶⁴⁶ (arginine finger), and (iii) D³⁹⁴RLLPDKAID⁴⁰³ and M⁷⁰²GARPM⁷⁰⁷ (sensor 2). Extensive functional studies of different AAA+ proteins demonstrated that several amino acid residues are critical for AAA+ ATPase activity (Wendler et al. 2012). The **Fig. 4c** (right part) denotes the conserved nucleotide interacting residues in the AAA+ module of ClpA in Xcc17, in which the effects of mutations with regard to oligomerization and hydrolytic activity have been tested in other AAA+ proteins (Wendler et al. 2012). As denoted **Fig. 4c** (right part), the residues in AAA+ module D1 are: (i) K223 (Walker A), (ii) E289 (Walker B), (iii) T326 (sensor 1), (iv) R342 and R343 (arginine finger), and (v) R395 (sensor 2), and K504 (Walker A), D567 (Walker B), N609 (sensor 1), R646 (arginine finger), and R705 (sensor 2) in the AAA+ module D2. Given the conserved sequence motifs and catalytic residues of the *X. campestris* pv. *campestris* ClpA to homologs from a diverse set of bacteria, it is suggested that the Xcc17 ClpA is a functional AAA+ ATPase.

ClpA is required for survival under heat-shock condition

To investigate the function of ClpS and ClpA in *X. campestris* pv. *campestris*, we proceeded to generate the mutants (*DclpS* and *DclpA*) and their complementary strains *DclpS*(pRKclpS) and *DclpA*(pRKclpA) as described in the **Materials and methods** section. Due to the observations that the expression of both *clpS* and *clpA* is induced by heat shock, it is reasonable to suggest a role of these proteins in stress adaptation. We first characterized the impact of *clpS* and *clpA* deletions in sensitivity to heat stress. Cell suspensions from tenfold serial dilutions of the *X. campestris* pv. *campestris* wild-type strain, *clpS* and *clpA* mutant strains, and their complementary strains were spotted on the LB agar plates and incubated either at 28 °C or 37 °C. Under physiological temperature (28 °C), all strains spotted at all densities grew similarly and displayed undistinguishable colony morphology (**Fig. 5**, left part). Under heat shock condition (37 °C), the Xcc17(pRK415), *DclpS*(pRK415), *DclpS*(pRKclpS) and *DclpA*(pRKclpA) displayed similar growth behavior whereas the growth of *clpA* mutant *DclpA*(pRK415) was inhibited (**Fig. 5**, right part). It is suggested that deletion of *clpS* does not affect sensitivity to heat stress under the assay condition, whereas the *clpA* gene is required for stress tolerance in *X. campestris* pv. *campestris*. Similar situations are observed in the *clpA* mutants of *B. susi* and *Salmonella* Typhimurium (Ekaza et al. 2000, Sangpuui et al. 2018). In *B. susi*, the *clpA* mutant showed reduced growth rates at elevated temperature (Ekaza et al. 2000). In *S. Typhimurium*, the *clpA* mutant was hypersusceptible to 42 °C exposure (Sangpuui et al. 2018). These observations contrast with results obtained for *E. coli*, in which the ClpA does not appear to be a heat-shock protein, and the *clpA* mutants grow well at temperatures between 25 and 42 °C (Katayama et al. 1988). Despite the fact that *E. coli* ClpA is not a heat-shock protein, the *clpA* mutant is defective for growth at 46 °C, and this protein appears to have a role in cellular recovery from transient incubation at 50 °C (Thomas and Baneyx 1998).

The Clp family protease has a multitude of functions in bacteria, such as protein quality control, stress tolerance, and virulence factor expression (Malik and Brotz-Oesterhelt 2017). In *S. Typhimurium*, the *clpA* mutant strain showed susceptibility to HOCl (Sangpuui et al. 2018). Recently, it is reported that *clpS* and *clpA* are important in enabling *E. coli* to grow during H₂O₂ stress (Sen et al. 2020). In *X. campestris* pv. *campestris*, ClpX and ClpP, other members of Clp family protein, are documented to be important for the

survival of this bacteria under various stresses, including temperature and puromycin (Li et al. 2020, Lo et al. 2020). The *X. campestris* pv. *campestris* ClpX also has a role in SDS tolerance (Lo et al. 2020). To examine whether *clpS* and *clpA* have a role in other stress tolerance, the sensitivities of *DclpS* and *DclpA* to H₂O₂, SDS, and puromycin were assessed. The *DclpS* and *DclpA* did not differ from the *Xcc17* in susceptibility to these stresses (data not shown). It is similar to the observation in *Acinetobacter baumannii*, in which deletion of *clpS* and *clpA* genes does not alter oxidative stress sensitivity (Belisario et al. 2021). Taken together, these results suggested that *clpX* is more important than *clpA* in the survival of *X. campestris* pv. *campestris* under H₂O₂, SDS, and puromycin treatment. We cannot exclude that ClpS and ClpA contribute to other stresses, further insight regarding this aspect has to be gained.

In AAA+ proteins with two AAA+ ATPase modules, such as ClpA, the contributions of both regions to ATP binding/hydrolysis differ (Bittner et al. 2016). Mutational and functional analyses have demonstrated that the two domains of ClpA of *E. coli* possess different functional roles: first AAA+ domain in ClpA is crucial for oligomerization while the second AAA+ domain is primarily responsible for ATP hydrolysis (Pak et al. 1999, Seol et al. 1995, Singh and Maurizi 1994), and the two domains operate independently even in the presence of ClpP or ClpS (Kress et al. 2009b). In *E. coli* ClpA, it is demonstrated that: (i) the domain 1 mutant (ClpA-K220Q) was unable to form a hexamer, whereas the comparable domain 2 mutant (ClpA-K501Q) associated into a hexamer, and (ii) ClpA-K220Q was defective in ATPase activity and in the ability to activate protein and peptide degradation by ClpP, and (iii) ClpA-K501Q had very low ATPase activity and a severe defect in activation of protein degradation, but it was able to activate ClpP to degrade a peptide (Singh and Maurizi 1994). To investigate whether the conserved motif observed in ClpA had any impact in the heat stress tolerance in *X. campestris* pv. *campestris*, we performed site-directed mutagenesis to construct mutated versions of ClpA. The most common mutated residues (K223 in AAA+ module 1 and K504 in module 2) were selected and exchanged to glutamine. As depicted in **Fig. 5** (left part), the *DclpA* complemented with mutated version pRKclpAK223Q or pRKclpAK504Q did not show significantly growth difference to other tested strains at 28 °C. However, introduction of pRKclpAK223Q or pRKclpAK504Q to *DclpA* cannot restore the growth behavior as those observed in *DclpA* complemented with the wild-type version pRKclpA under heat shock condition **Fig. 5** (right part). It is suggested that the putative residues K223 and K504 are associated with full function of ClpA in *X. campestris* pv. *campestris* stress tolerance. The growth of *clpA* mutant complemented with pRKclpAK223Q was similar to those mutant complemented with pRK415 (empty vector), whereas it is worth to noted that growth was prominent in the areas spotted with 5×10^8 cells of *DclpA*(pRK415), *DclpA*(pRKclpA), and *DclpA*(pRKclpAK223Q), but not in areas spotted with the same concentration of *DclpA*(pRKclpAK504Q) at 37 °C (**Fig. 5**, right part). It is not clear why introduction of ClpA with K504 mutation led to the *clpA* mutant a failure of forming colony under heat stress treatment. Introduction of pRKclpAK504Q seems to be detrimental to *X. campestris* pv. *campestris* and both predicted AAA+ modules in ClpA seems to possess different roles in this bacteria. The impacts of pRKclpAK504Q in the growth of *clpA* mutant and the function of each module of ClpA require further study.

The *clpA* mutant shows differential protein expression at high temperature

ClpA can function as a molecular chaperon preventing aggregation, and has a role in refolding and remodeling of proteins (Hoskins et al. 2001, Pak and Wickner 1997, Suzuki et al. 1997, Wawrzynow et al. 1996, Wickner et al. 1994). The impact of growth in the *clpA* mutant at 37 °C suggested the participation of ClpA in preventing the accumulation of heat-inactivated and aggregate proteins in *X. campestris* pv. *campestris*. To test whether *clpA* deletion would alter the protein expression profile and affect the aggregation of proteins in heat-shocked cell, we grown the mutant and parental strains at 28 °C or 37 °C, then the protein samples were prepared and fractionated by SDS-PAGE as described in the **Material and methods** section. When cultures were grown at 28 °C, the protein profiles obtained without Triton X-100 treatment were not significantly different between the parental strain and the *clpA* mutant (**Fig. 6, lanes 1 and 5**), similar patterns were observed when Triton X-100 treatment was included (**Fig. 6, lanes 2 and 6**). When the bacteria were cultured at 37 °C, several protein bands differ between the parental strain and the *clpA* mutant, despite wholesale aggregation of proteins does not occur in the *clpA* mutant. In the absence of Triton X-100 treatment, the band I was observed in wild-type Xcc17 and was missing in the *clpA* mutant D*clpA* (**Fig. 6, lanes 3 and 7**). When Triton X-100 was included, the band II was observed in Xcc17 but not in D*clpA*, and bands III and IV were observed in D*clpA*, which were too faint to be visible in Xcc17 (**Fig. 6, lanes 4 and 8**). Further studies are required to identify these mainly altered protein bands to elucidate the significance of the change in their amounts. In *E. coli*, the amount of protein aggregation was not significantly increased in *clpA* mutant compared to the wild-type cells (Dogan et al. 2002), whereas in *S. Typhimurium*, the *clpA* mutant revealed greater amounts of protein aggregates than in the wild-type strain (Sangpui et al. 2018).

ClpA is required for full virulence

As Clp family proteins are documented to be important for bacterial pathogenesis in several bacterial species (Brotz-Oesterhelt and Sass 2014), we wished to test whether this was also the case in *X. campestris* pv. *campestris*. To determine whether *clpS* and *clpA* is involved in the pathogenicity, the virulence of *X. campestris* pv. *campestris* was tested on the host plant cabbage by leaf-clipping inoculation. The inoculants for the pathogenicity test included Xcc17(pRK415), D*clpS*(pRK415), D*clpS*(pRK*clpS*), D*clpA*(pRK415), and D*clpA*(pRK*clpA*). The lesion length caused by the wild-type strain was approximately 2 cm at 14 days post inoculations, and the *clpS* mutant and its complementary strain caused virulence symptoms similar to those of the wild-type (**Fig. 7**). Although the *clpA* mutant could cause disease it was significantly attenuated compared with the wild type, and the complemented strain restored disease symptoms toward the wild-type phenotype (**Fig. 7**). These results indicate that *clpS* is not related to virulence and *clpA* is required for the full virulence of *X. campestris* pv. *campestris*. It is similar to the observations reported in *Pseudomonas aeruginosa*, in which *clpA* mutant but not *clpS* mutant exhibits virulence-attenuated phenotypes (Feinbaum et al. 2012). It has been suggested that disruption of ClpA function is responsible for the altered pathogenesis of *Ralstonia solanacearum* (Lin et al. 2008). A recent study in *A. baumannii* found that both *clpS* and *clpA* were important for virulence (Belisario et al. 2021).

In *Xanthomonas* species, it is reported that successful infection often depend on an arsenal of virulence factors, such as adhesins, degradative enzymes, and extracellular polysaccharides (Buttner and Bonas 2010, Chan and Goodwin 1999, Denance et al. 2016, Dow et al. 2003, Tang et al. 2021). To test whether the reduced virulence in the *clpA* mutant was correlated to a reduced production of these pathogenicity factors, we investigated the contribution of *clpA* to bacteria attachment and extracellular enzyme production. No considerable differences in both bacterial attachment and extracellular enzyme production were observed between the *clpA* mutant and the parental strain (data not shown). In *A. baumannii*, both *clpS* and *clpA* were essential for biofilm formation (Belisario et al. 2021). In *X. campestris* pv. *campestris*, *clpX* was also reported to be required for bacterial attachment (Lo et al. 2020). According to the *X. campestris* pv. *campestris* genome annotation, numerous virulence determinants are associated with bacterial pathogenesis, *clpA* might impact in other unidentified factor(s) which remains further evaluation.

Together with above results concerning ClpS, it is observed that mutation of *clpS* has no effect on stress tolerance and virulence, even its expression is heat inducible. Whether this gene has other physiological role(s) in *X. campestris* pv. *campestris* remains to be investigated. In the case of ClpA, even mutation of this gene resulted in growth defect at high temperature and attenuated pathogenicity, the virulence-related factors and a range of stresses did not alter significantly. Compared to the previous finding regarding ClpX, the other member of Clp ATPase, mutation of *clpX* caused pleiotropic effects, including attachment, virulence, and stress tolerance (Lo et al. 2020). It is reasonable to suggest that ClpX plays a more essential role in *X. campestris* pv. *campestris*. Owing the observations that (i) *clpA* expression is heat inducible, (ii) mutation of *clpA* is impact in growth at high temperature, and (iii) *clpA* mutant presents different protein pattern under heat treatment, it could be that ClpAP protease evolved to deal with the increased need for proteolysis caused by increased denaturation or aggregation, under heat shock conditions. It remains unknown whether ClpA can associated with ClpP, also the role of ClpS as an modulator affecting the function of ClpA is still unclear in *X. campestris* pv. *campestris*. Further clarification the nature of the ClpS, ClpA, and ClpP interaction can provide novel insights and gain a more comprehensive description of these proteins.

Conclusion

In this study, we investigated the transcription and function of *clpS* and *clpA* in *X. campestris* pv. *campestris*. First, upstream region analysis together with reporter assay indicated that (i) putative σ^{32} -dependent sequences are present in the upstream region of *clpS* and *clpA*, (ii) both *clpS* and *clpA* contain their own promoter and are transcript independently, and (iii) the *clpS* and *clpA* promoters are induced approximately two-fold over control levels in response to heat stress. Second, through sequence comparison and homology modeling, the conserved sequence motifs were identified and the predicted three-dimensional structures of ClpS and ClpA were determined. Third, by combining insertional inactivation and phenotypic evaluation, it was revealed that mutation of *clpA* but not *clpS* causes defect in heat stress tolerance and pathogenicity, and the conserved residues of Walker A motif of the AAA+

ATPase module of ClpA are demonstrated to be required for ClpA function in heat tolerance. Finally, SDS-PAGE analysis revealed that the *clpA* mutant shows different protein profiles in response to heat shock treatment.

The Clp family proteins are found in most domains of life and the functional importance of these proteins varies from organism to organism. The genome of *X. campestris* pv. *campestris* is predicted to encode several common Clp family proteins, such as ClpS, ClpA, ClpX, ClpP, ClpQ, and ClpY (da Silva et al. 2002, Liu et al. 2015, Qian et al. 2005, Vorholter et al. 2008). While these proteins have been well-described in other organism, the function of these proteins are poorly understood in *X. campestris* pv. *campestris*. To date, only two (ClpX and ClpP) were found in the literature documenting their function in *X. campestris* pv. *campestris*. Both, ClpX and ClpP, are important in extracellular enzyme production, virulence, as well as heat and puromycin tolerance, suggesting the importance of ClpXP in pathogenesis and environmental adaptation (Li et al. 2020, Lo et al. 2020). The *clpX* mutant of *X. campestris* pv. *campestris* also exhibits reduced bacterial attachment and results in increased sensitivity to SDS (Lo et al. 2020). The expression of *X. campestris* pv. *campestris clpP* is shown to be heat inducible (Li et al. 2020). Combine with the finding we have presented here, there is a growing body of evidence that the Clp family proteins can be regarded as key virulence factors in *X. campestris* pv. *campestris*. They are plausible candidates for the development of new therapeutics to combat this phytopathogen.

Declarations

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Tables

Table 1. PCR primers used in this study

Primer	Sequence ^a	Direction and purpose ^b
35PstI	5¢- <u>CTCGAG</u> CAGGCTTTTCGTCCGGCT-3¢	F, Promoter analysis
476XbaI	5¢- <u>TCTAGAT</u> TATACGGTCCCGCGCCCTC-3¢	R, Promoter analysis
401HindIII	5¢- <u>AAGCTT</u> CCGGCCGGAGAACAGGCGTA-3¢	F, Complementation
946XbaI	5¢- <u>TCTAGAT</u> TGGTAACTCCGAACGGCTGT-3¢	R, Promoter analysis, mutant construction and complementation
621PstI	5¢- <u>CTGCAG</u> CAGTTCTTCAACCTGGA-3¢	F, Promoter analysis and mutant construction
899HindIII	5¢- <u>AAGCTT</u> CCCAACAAAAGCCGCATATTG-3¢	F, Mutant construction and complementation
3232XbaI	5¢- <u>TCTAGAC</u> GATCAATCAACCGTAGCCG-3¢	R, Mutant construction and complementation
223F	5¢-ccggcgtgggc <u>cag</u> accgcgatc-3¢	F, site-directed mutagenesis
223R	5¢-gatcgcggt <u>ctg</u> gccacgccgg-3¢	R, site-directed mutagenesis
504F	5¢-gaccggtgtgggc <u>cag</u> accgaggtcac-3¢	F, site-directed mutagenesis
504R	5¢-gtgacctcggt <u>ctg</u> gccacaccggtc-3¢	R, site-directed mutagenesis

^a: Added restriction enzyme sites are underlined. The mutated bases are in boldface and underlined

^b: F, forward direction, R, reverse direction

Figures

Figure 1

Comparative analysis of the organization of the *clpS-clpA* regions in the genome of the *X. campestris* pv. *campestris* and *E. coli*. The *X. campestris* pv. *campestris* strain 17 (Xcc 17) and the compatible regions in *E. coli* strain K-12 (Eco K-12) are shown. The arrows show the locations and orientations of the genes, and lines show the intergenic regions. The percentage values refer to the amino acid identity between each two homologous proteins. The inverted triangles indicate the position of pOKclpS and GmR cartridge in the *clpS* and *clpA* mutants. The lower elements indicate the lengths and locations of PCR-amplified fragments used for expression, mutation, and complementation analysis. The construction of the reporter constructs (pFYclpS and pFYclpA) and the complementation plasmids (pRKclpS, pRKclpA, pRKclpAK223Q, and pRKclpAK504Q) is described in Materials and Methods. The primers used to construct these plasmids are shown below the solid lines

Figure 2

Expression of *clpS* and *clpA* in *X. campestris* pv. *campestris*. The wild-type strain Xcc17 harboring the reporter construct pFYclpS (a) or pFYclpA (b) was cultured in LB medium. The open bars and filled bars represent β -galactosidase activities (Miller units) of the reporter strain cultured at 28 °C and 37 °C, respectively. Results are shown as an average of triplicate measurements. Error bars indicate standard deviations. Significance was tested by Student's t test (* indicates significance at $p < 0.05$)

Figure 3

Domain organization prediction (a) and model of three-dimensional structure (b) of *X. campestris* pv. *campestris* ClpS. a. The predicted ClpS domain (PF02617) is shown and is drawn approximately to scale. b. The N- and C-terminal ends are indicated (N' and C', respectively). The predicted amino acid residues responsible for ClpS binding to the contact A and contact C of the N-domain of ClpA are revealed by yellow shading and green shading, respectively

Figure 4

Domain organization prediction (a) and model of three-dimensional structure (b and c) of *X. campestris* pv. *campestris* ClpA. a. The predicted N-domain, AAA+ module D1 and AAA+ module D2 are shown and are drawn approximately to scale. The large and small subdomain of each AAA+ module is shown in ellipse and hexagonal, respectively. Typical elements of the AAA+ module are highlighted in color: Walker A (red), Walker B (green), sensor 1 (orange), arginine finger (purple), and sensor 2 (brown). b. Schematic presentation of the structure of ClpA. The N- and C-terminal ends are indicated (N' and C', respectively). The N-domain and the two AAA+ ATPase domain (D1 and D2) are highlighted with a square in green and ovals in blue, respectively. c. Close-up view of the site in a similar orientation as in part (b) in a stick and colored representation. Left part (N-domain): The conserved residues in the contact A and contact C of the N-domain of ClpA are revealed by yellow and green shading, respectively. Amino acid residues have potential roles in Zn²⁺ binding and peptide binding are shown in blue and orange shading, respectively. The colors of each corresponding residues are also shown in a. Right part (ATPase domain): The symbols in front of each amino acid residues denote the highly conserved and postulated amino acid residues required for ClpA function. Asterisks (*) and triangles (▲) denote the residues conserved in

Walker A and Walkler B. Diamonds (◆) and squares (■) denote the residues conserved in Sensor 1, and Sensor 2. Filled circles (●) present the conserved residues in Arginine finger. The amino acid residues characterized in the present study are underlined

Figure 5

Effects of *clpS* and *clpA* mutation on cell growth under heat shock treatment. Overnight cultures of the indicated strains were spotted on LB agar plates with tenfold serial dilutions. Plates were incubated at 28 °C or 37 °C for 4 days prior to imaging. The inoculating concentrations of bacterial cells of 100 refer to 5 x 10⁸ cells/mL. The experiment was carried out least three times and similar results were obtained

Figure 6

Effects of *clpA* mutation on protein production profile of *X. campestris* pv. *campestris*. The cells were cultured in XOLN medium supplemented with 2% glycerol for 24 h at 28 °C or 37 °C. Protein samples from the indicated strains were separated by SDS-PAGE on a 15% polyacrylamide gel and stained with Coomassie blue. The same protein concentration of each lysate was loaded in the wells. The experiment was performed least three times and similar results were obtained. The filled triangles (◀) and open triangles (◁) point out protein bands upregulated and downregulated after *clpA* mutation, respectively

Figure 7

Effects of *clpS* and *clpA* mutation on the virulence of *X. campestris* pv. *campestris* to cabbage. Black rot symptoms caused by the indicated strains on inoculated leaves of the cabbage. Lesion lengths were measured and images were taken at 14-day post-inoculation. Scale bars = 1 cm. Similar results were obtained at least three times. Values of average lesion lengths (cm) are presented as mean $\pm$ standard deviation. Values marked with different letters indicate significant difference ($p < 0.05$)